

ORIGINAL ARTICLE

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Atorvastatin induces associated reductions in platelet P-selectin, oxidized low-density lipoprotein, and interleukin-6 in patients with coronary artery diseases

Received: December 26, 2006 / Accepted: December 28, 2007

Abstract The development and progression of atherosclerosis comprises various processes, such as endothelial dysfunction, chronic inflammation, thrombus formation, and lipid profile modification. Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that have pleiotropic effects in addition to cholesterol-lowering properties. However, the mechanisms of these effects are not completely understood. Here, we investigated whether atorvastatin affects the levels of malondialdehyde-modified low-density lipoprotein (MDA-LDL), an oxidized LDL, the proinflammatory cytokine interleukin-6 (IL-6), or platelet P-selectin, a marker of platelet activation, relative to that of LDL cholesterol (LDL-C). Forty-eight patients with coronary artery disease and hyperlipidemia were separated into two groups that were administered with (atorvastatin group) or without (control group) atorvastatin. The baseline MDA-LDL level in all participants significantly correlated with LDL-C ($r = 0.71$, $P < 0.01$) and apolipoprotein B levels ($r = 0.66$, $P < 0.01$). Atorvastatin (10 mg/day) significantly reduced the LDL-C level within 4 weeks and persisted for a further 8 weeks of administration. Atorvastatin also reduced the MDA-LDL level within 4 weeks and further reduced it over the next 8 weeks. Platelet P-selectin expression did not change until 4 weeks of administration and then significantly decreased at 12 weeks, whereas the IL-6 level was gradually, but not significantly, reduced at 12 weeks. In contrast, none of these parameters significantly changed in the control group within these time frames. The reduction (%) in IL-6 between 4 and 12 weeks after atorvastatin administration significantly correlated with that of MDA-LDL and of platelet P-selectin ($r = 0.65$, $P < 0.05$ and $r = 0.70$, $P < 0.05$, respectively). These results suggested that the positive effects of atorvastatin on the LDL-C oxidation,

platelet activation and inflammation that are involved in atherosclerotic processes are exerted in concert after lowering LDL-C.

Key words Statin · Pleiotropic effects · Oxidative stress · Platelet activation · Inflammation

Introduction

Oxidized low-density lipoprotein (LDL) is believed to play a key role in the pathogenesis of coronary atherosclerosis, which is a multifactorial condition that includes endothelial dysfunction, chronic inflammatory responses, plaque instability, and thrombus formation. The oxidation of LDL alters its native properties and allows scavenger receptors to incorporate it into macrophages where it negatively affects various functions in the vascular walls, such as the inhibition of endothelial nitric oxide (NO) production, endothelial apoptosis, and the proliferation of smooth muscle cells.¹ Malondialdehyde-modified LDL (MDA-LDL; an oxidized LDL) has been isolated from the sera of patients with coronary artery diseases (CAD)² and levels are elevated in patients with CAD, diabetes mellitus (DM), and hyperlipidemia.^{3–6} Furthermore, circulating levels of MDA-LDL correlate with the intima-media thickness of carotid arteries.⁷ These facts suggest that MDA-LDL could be a good indicator of atherosclerosis.

Platelets are prominent components of thrombi and their activation is a key step in the progression of atherosclerosis. Indeed, atherectomy in patients with myocardial infarction has revealed that activated platelets are located close to atherosclerotic plaque.⁸ P-selectin, a marker of platelet activation, is related to the magnitude of the distribution of atherosclerosis in patients with coronary artery disease and/or peripheral arterial disease.⁹ Furthermore, P-selectin expression on platelets is elevated in patients with atherosclerotic ischemic stroke¹⁰ or hypercholesterolemia.¹¹ Johnson et al.¹² also demonstrated that fatty streaks were smaller in the aortae of mice that were P-selectin-deficient

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than in those that were P-selectin-positive. Thus, platelet P-selectin expression might be an important factor in the atherothrombotic process.¹³

The proinflammatory cytokine, interleukin-6 (IL-6), is a powerful inducer of C-reactive protein (CRP),¹⁴ which is a useful predictor of coronary artery events.¹⁵⁻¹⁷ Reports indicate that increased levels of circulating IL-6 are also related to a high risk of mortality due to myocardial infarction and coronary heart disease.^{18,19} Interleukin-6 can interfere with lipid metabolism by down-regulating lipoprotein lipase and modulating the immune response of vascular cells.²⁰⁻²²

Although oxidized-LDL, platelet P-selectin, and IL-6 contribute to the development and progression of atherosclerosis as noted above, associations among these factors remain obscure. Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that can reduce the likelihood of cardiovascular events independently of their ability to lower cholesterol; that is, statins are pleiotropic.²³⁻²⁶ The molecular mechanisms of pleiotropic effects involve increased endothelial NOS expression through inhibiting geranylgeranylation of the small GTP-binding protein, Rho,²⁷ a post-translational activator of the phosphatidylinositol 3-kinase/Akt pathway,²⁸ and interaction with heat shock protein 90.²⁹ Such modulation of the intracellular signaling pathway leads to improved endothelial and platelet function and suppresses vascular inflammation.³⁰⁻³² However, whether these beneficial properties of statins are inter-related remains unknown, particularly in the clinical setting.

The present study found that atorvastatin reduced the levels of MDA-LDL and P-selectin on the surface of platelets and of IL-6 in patients with CAD and hyperlipidemia. These changes were inter-related at 4 and 12 weeks of atorvastatin administration, and subsequently associated with decreased cholesterol values.

Materials and methods

We enrolled 48 consecutive patients with hypercholesterolemia and CAD confirmed by coronary angiography

between August 2000 and August 2001. The patients were evaluated according to the Japanese Atherosclerosis Society guidelines for the diagnosis and treatment of atherosclerotic cardiovascular diseases, and then randomly separated into one group that received 10 mg of oral atorvastatin daily for 12 weeks with diet therapy and another (control) that received only diet therapy for 12 weeks. Table 1 shows the characteristics of the patient groups. All were stabilized at the start of this study, had been administered with aspirin as an antiplatelet agent and had not changed other prescribed medications such as ACE inhibitors, β -blockers, Ca-channel antagonists, or diuretics. Four patients were excluded from the atorvastatin group because of side effects, liver dysfunction, and elevated creatinine kinase. All patients provided written, informed consent and all procedures followed institutional guidelines. Appropriate treatment was applied, including the administration of statins to the control group after completion of this study. Blood samples were collected from both groups immediately before, and at 4 and 12 weeks after the start of the study.

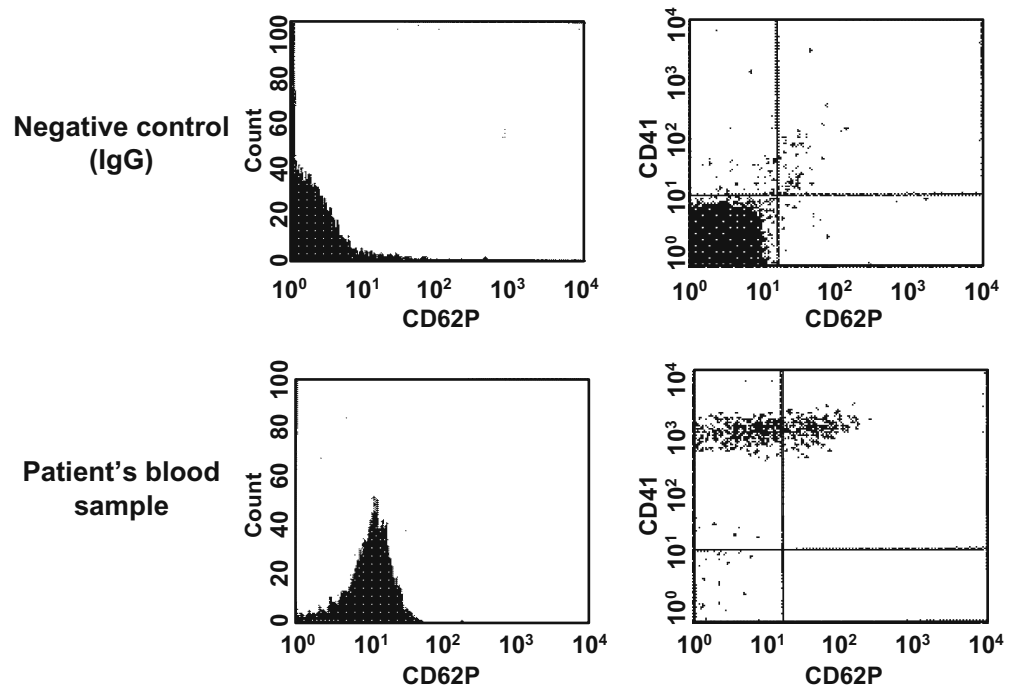
The level of P-selectin on platelet membranes was analyzed by whole-blood flow cytometry essentially as described.³³ In brief, peripheral blood samples were collected from resting patients without venous return occlusion using a tourniquet, into K3 EDTA vacutainers. Thereafter, they were fixed in 1.0% formaldehyde/phosphate-buffered saline (PBS) for at least 2 h at 4°C, washed twice with 0.1% sodium azide/PBS, and incubated with monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-CD62P and phycoerythrin (PE)-conjugated anti-CD41 for 15 min at room temperature in the dark. The surface expression of CD62P (P-selectin) and CD41 (GP IIb/IIIa), a specific platelet antigen was determined by flow cytometry using a FACSCAN (Becton, Franklin Lakes, NJ, USA). The platelets were gated by CD41 staining and transferred to FL 1 (CD62P-FITC) on the *x* axis and FL 2 (CD41-PE) on the *y* axis in the histogram shown in Fig. 1. Platelet P-selectin levels are expressed as %CD62P positive cells among 50000 platelets (CD41-positive cells). Nonspecific immunostaining for CD62P or CD41 was determined using

Table 1. Characteristics of patients at baseline

	Atorvastatin	Control	<i>P</i> value
Number	25	23	
Sex (male/female)	14/11	13/10	NS
Age (years)	68.9 ± 8.2	66.2 ± 6.8	NS
BMI (kg/m ²)	25.8 ± 3.1	23.9 ± 5.6	NS
Total cholesterol (mg/dl)	215.2 ± 22.6	211.7 ± 10.8	NS
HDL-C (mg/dl)	51.9 ± 21.5	46.2 ± 7.4	NS
LDL-C (mg/dl)	135.8 ± 19.4	132.2 ± 13.7	NS
TG (mg/dl)	137.0 ± 62.4	165.1 ± 45.8	NS
Hypertension (%)	53	43.5	NS
Diabetes mellitus (%)	20	26	NS
Smoking (%)	36	21.7	NS
Underlying diseases: AP/MI	12/13	15/8	NS

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; AP, angina pectoris; MI, myocardial infarction; NS, not significant

Fig. 1. Representative flow cytometry histograms and dot plots. **Upper panels** Negative control (IgG). **Lower panels** Patient's blood sample. Expression of platelet P-selectin was determined as CD62P-positive cells gated by CD41-positive staining. All data are results of 50000 total events



an irrelevant isotype IgG-FITC or -PE conjugated as a negative control. Fasting venous blood samples were collected between 06:00 and 08:00 h to measure other parameters. Sera were analyzed for total cholesterol, LDL cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein B (apo-B), apolipoprotein AI (apo-AI), and creatinine kinase (CK) levels, whereas renal profiles, liver function, and prothrombin time (PT) were determined using routine laboratory techniques at our hospital. Levels of MDA-LDL were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) (SRL, Tokyo, Japan). Levels of serum IL-6 were measured using a sandwich ELISA (Fujirebio, Tokyo, Japan). The percentage change (% change) in levels of parameters was calculated by subtracting the value obtained before, from that obtained after two examinations and dividing the result by the levels obtained before.

Statistical analysis

All results are expressed as mean \pm SD. Differences between two groups were analyzed using Student's *t*-test or the Mann-Whitney test. Changes in levels of parameters before, and at 4 and 12 weeks after treatment were compared using repeated-measures analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Correlations between two variables are described using Pearson's or Spearman's rank correlation. All data were statistically analyzed using SPSS II version 11 software (SPSS, Chicago, IL, USA). A *P* value of less than 0.05 was considered significant.

Results

Relationships among baseline MDA-LDL, platelet P-selectin, IL-6, and lipid profiles

The baseline lipid profile of the patients was as follows (mg/dl): 213.5 ± 17.9 total cholesterol, 135.1 ± 16.8 LDL-C, 150.4 ± 56.4 TG, and 49.2 ± 16.4 HDL-C (mean \pm SD), indicating hyper-LDL-cholesterolemia. The levels of MDA-LDL, P-selectin, and IL-6 were not as elevated as those in previous reports,^{3,34-36} suggesting that CAD was stable in our patients. In this setting, MDA-LDL significantly correlated with LDL-C ($r = 0.71$, $P < 0.01$) and apo-B ($r = 0.66$, $P < 0.01$), but not with TG and HDL-C (Fig. 2). No relationships among baseline P-selectin, MDA-LDL, and IL-6 were identified (Fig. 3).

Effects of atorvastatin on MDA-LDL, P-selectin, IL-6, and lipid profiles at 4 and 12 weeks after administration

Table 1 shows that age, sex, body mass index (BMI), lipid profiles, and complications of hypertension and diabetes mellitus did not significantly differ between the two groups of patients. Atorvastatin (10 mg/day) significantly reduced LDL-C levels after 4 and 12 weeks of administration (28.2% and 26.7% reduction, respectively). In contrast, the level did not change in the control group after dietary therapy over the same period (Fig. 4). Furthermore, levels of HDL-C and TG also significantly differed at 4 and 12 weeks of administration compared with the control group (Fig. 4).

Fig. 2. Relationship between malondialdehyde-modified low-density lipoprotein (MDA-LDL) and lipid profile at baseline. Relationships between MDA-LDL and low-density lipoprotein cholesterol (LDL-C) (upper left), apolipoprotein B (apo-B) (upper right), triglycerides (TG) (lower left), and high-density lipoprotein cholesterol (HDL-C) (lower right)

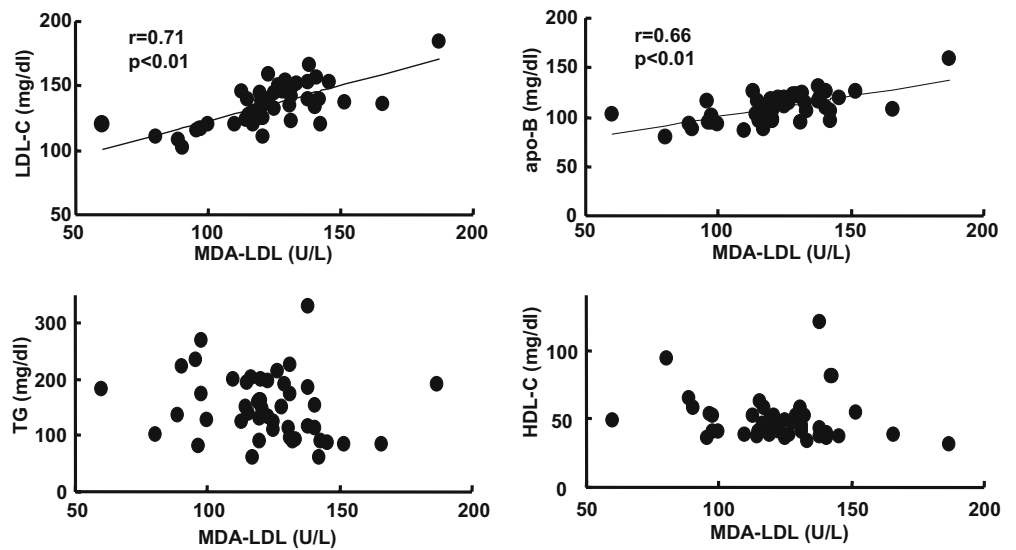


Fig. 3. Relationships between platelet P-selectin and MDA-LDL and interleukin-6 (IL-6). **Left** Correlation between P-selectin and MDA-LDL. **Right** Correlation between P-selectin and IL-6

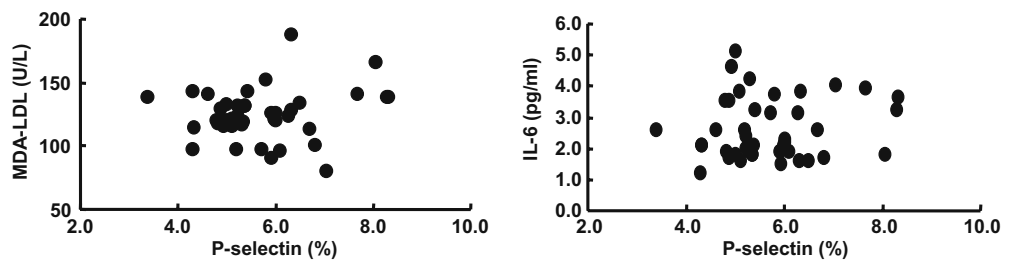
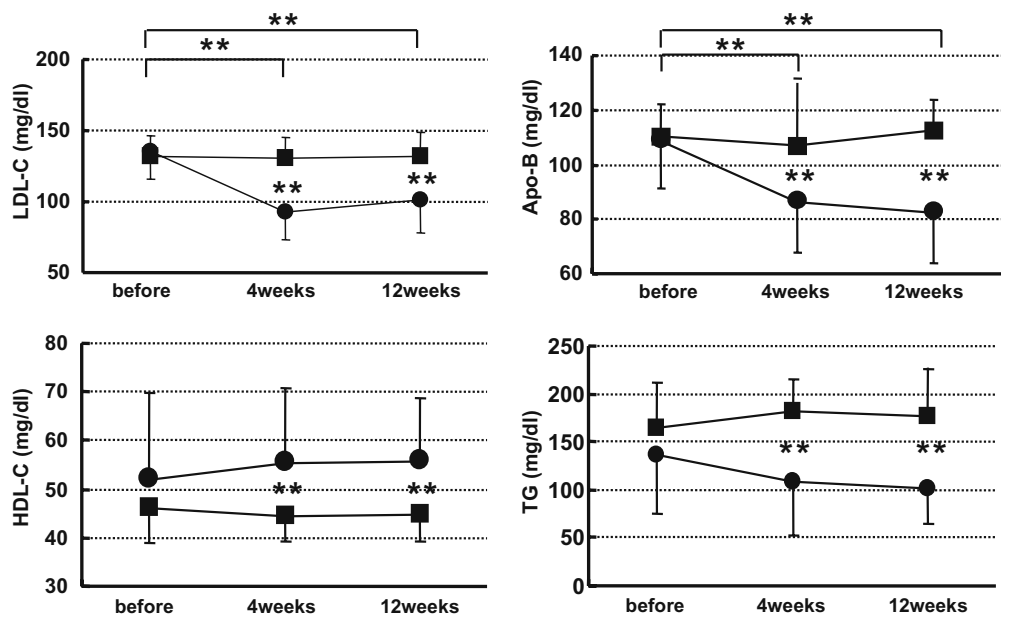


Fig. 4. Changes in lipid profile before, and after atorvastatin for 4 and 12 weeks. *Closed circles and squares*, atorvastatin and control groups, respectively. Changes in MDA-LDL (upper left), apo B (upper right), TG (lower left), and HDL-C (lower right). * $P < 0.05$, ** $P < 0.01$



The MDA-LDL concentration decreased after 4 and 12 weeks of atorvastatin administration compared with that before treatment, but did not significantly differ from that of the control group ($P = 0.08$ and $P = 0.05$ at 4 and 12 weeks, respectively) (Fig. 5). The platelet P-selectin level was not obviously changed after 4 weeks of atorvastatin administration, but significantly decreased after 12 weeks

(Fig. 5). The levels of P-selectin in the control group before and at 4 weeks after administration were lower than those of the atorvastatin group, and dietary therapy did not significantly affect the levels until 12 weeks. In contrast, PT, an indicator of the extrinsic coagulation pathway, did not significantly change in either group (Fig. 5). Atorvastatin tended to gradually reduce the IL-6 concentration over 12

Fig. 5. Changes in MDA-LDL, IL-6, P-selectin, and prothrombin time (PT) before and after atorvastatin for 4 and 12 weeks. Closed circles and squares indicate atorvastatin and control groups, respectively. Upper left, MDA-LDL; upper right, IL-6; lower left, P-selectin; lower right, PT. * $P < 0.05$; ** $P < 0.01$

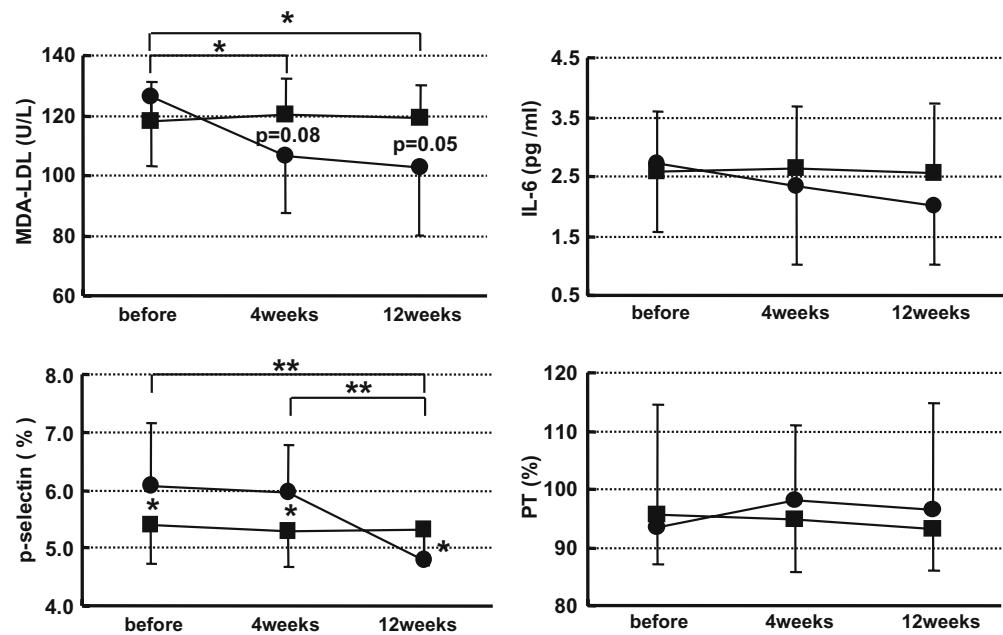


Table 2. Correlations among % changes in MDA-LDL, P-selectin, IL-6, and LDL-C

	Correlation coefficient	P value
% Change between before and 4 weeks later		
LDL-C vs MDA-LDL	0.48	0.09
LDL-C vs P-selectin	0.42	0.13
LDL-C vs IL-6	0.05	0.45
MDA-LDL vs P-selectin	-0.17	0.33
MDA-LDL vs IL-6	-0.28	0.24
P-selectin vs IL-6	0.18	0.32
% Change between 4 and 12 weeks later		
LDL-C vs MDA-LDL	0.20	0.31
LDL-C vs P-selectin	-0.05	0.42
LDL-C vs IL-6	0.40	0.16
MDA-LDL vs P-selectin	0.47	0.11
MDA-LDL vs IL-6	0.65	0.04
P-selectin vs IL-6	0.70	0.03
% Change between before and 12 weeks later		
LDL-C vs MDA-LDL	0.53	0.06
LDL-C vs P-selectin	0.31	0.32
LDL-C vs IL-6	0.22	0.48
MDA-LDL vs P-selectin	0.41	0.14
MDA-LDL vs IL-6	0.27	0.37
P-selectin vs IL-6	0.29	0.35

LDL-C, low-density lipoprotein cholesterol; MDA-LDL, malondialdehyde-modified LDL; IL, interleukin

weeks, but the difference was not significant, which might be due to high variance (Fig. 5).

To investigate whether the changes induced by atorvastatin are interrelated, or are related to its LDL-lowering effect, we analyzed correlations as % change among MDA-LDL, P-selectin, IL-6, and LDL-C. Table 2 shows that the % change of MDA-LDL tended to positively correlate with that of LDL-C ($r = 0.48$, $P = 0.09$) between before and at 4 weeks after atorvastatin therapy. At between 4 and 12 weeks after treatment, correlations between MDA-LDL and IL-6 and between P-selectin and IL-6 were significant, but none of them correlated with LDL-C. No serious CAD

complications such as acute coronary syndrome, heart failure, fatal arrhythmia, or death occurred during this study.

Discussion

The present study shows that MDA-LDL correlated with LDL-C at baseline. We also showed that atorvastatin induced a change in the level of MDA-LDL at between 4 and 12 weeks after administration that was associated with changes in IL-6, but not in LDL-C levels. Moreover, the change in platelet activation detected by P-selectin expression on the surface was related to that of IL-6.

The oxidative modification of LDL-C is believed to play an important role in the progression of atherosclerosis.³⁷ Tanaga et al.⁶ demonstrated that the level of MDA-LDL is higher in patients with than without CAD, and correlated with LDL-C, TG, HDL-C, as well as with LDL particle size. The correlation of LDL with MDA-LDL in the present study was consistent with their findings. However, we could not find any correlation between TG and HDL. The lipid profiles including LDL-C, TG and HDL in our patients were slightly worse than theirs. Differences in patient characteristics and numbers might have contributed to the discrepancy. After atorvastatin administration, MDA-LDL tended to fall for 12 weeks, and the % change tended to correlate with that of LDL between before and at 4 weeks and significantly correlated with changes of IL-6 between 4 and 12 weeks. These results suggested that atorvastatin regulates oxidation partially through lowering LDL, and then by affecting inflammatory reactions in damaged vessels independently of this property. Tamura et al.³ described that the removal of substantial amounts of "aged" LDL, which is prone to oxidation in the circulation, through LDL receptor activity up-regulated by statins could contribute to

reducing the susceptibility of “new” LDL in the circulation to oxidation, resulting in a reduced level of circulating MDA-LDL. Statins and/or their metabolites have antioxidant effects that are markers of both a reduction in generalized oxidative stress and of LDL susceptibility to oxidation.³⁸ Massy et al.³⁹ demonstrated that oxidized as well as native LDL stimulates IL-6 mRNA expression and secretion in human mesangial cells, a process that lovastatin (5 μ M) can suppress within 16 h. The acute phase inflammatory protein, CRP, which is produced by IL-6 stimulation in hepatocytes, also positively correlates with oxidized LDL, and both CRP and IL-6 are indicators of event-free survival in patients with unstable angina.³⁵ Moreover, Sugiyama et al.⁴⁰ demonstrated that 4 weeks of treatment with 10 mg/day of atorvastatin significantly decreased CRP levels in hyperlipidemic patients. We did not find here that 10 mg/day of atorvastatin significantly reduced IL-6. The MIRACL study found that atorvastatin at 80 mg/day for 16 weeks reduces CRP but does not significantly reduce IL-6. This might be due to the greater diurnal variability and shorter half-life of IL-6 compared with CRP.³⁴ Marz et al.⁴¹ also found that statins significantly lower CRP but not IL-6 and inferred that statins lower CRP by interfering with the regulation of hepatic CRP production by IL-6 rather than by directly modulating inflammatory processes in vessel walls. Therefore, changes should be more obvious if CRP is measured as a marker of inflammation.

Atorvastatin in our patients did not obviously alter the level of P-selectin on the platelet surface until 4 weeks of administration, and then significantly reduced the amount thereafter up to 12 weeks of administration, which was associated with a reduction in IL-6. Several reports have shown that statins reduce the expression of P-selectin in patients with hypercholesterolemia and/or atherothrombosis.^{11,13,36,42–44} Significant effects start to appear from within 9 days to 6 months, which might be due to different study protocols, different types of statins, and the patient population. Others have examined soluble P-selectin as a marker of platelet activation. Here, we examined P-selectin expression on the surface of platelets by flow cytometry, because soluble P-selectin in plasma samples originates not only from platelets but also endothelium. Gurbel et al.⁴⁵ found that soluble and platelet P-selectin did not correlate in 300 patients with chest pain. Therefore, flow cytometry is a reliable and sensitive method of detecting platelet activation.⁴⁶ The changes in platelet P-selectin and IL-6 between 4 and 12 weeks after atorvastatin administration were significantly associated. Interleukin-6 is thought to be not only proinflammatory but also procoagulant, stimulating the synthesis of fibrinogen, plasminogen-activator inhibitor type I, and tissue factor.⁴⁷ Platelet activation determined as platelet P-selectin in response to thrombin and platelet activating factor (PAF) is enhanced in dogs treated with IL-6.⁴⁸ Another proinflammatory cytokine, tumor necrosis factor (TNF)- α , also promotes a procoagulant state by inhibiting the synthesis of anticoagulant protein C⁴⁹ and by eliciting tissue factor production on the endothelium and monocytes, thereby stimulating thrombin and fibrin formation.⁵⁰ Furthermore, fluvastatin (80 mg/day for 12 weeks) decreased

the level of IL-6 and TNF- α in patients with chronic heart failure.⁵¹ Thus, atorvastatin can modulate the expression of IL-6, which might have interacted with platelet activation after reducing LDL-C in the present study.

The significant correlation between MDA-LDL with IL-6 and the tendency toward a positive correlation between MDA-LDL and P-selectin induced by atorvastatin indicate that LDL-C oxidation, inflammation, and platelet activation interact and that atorvastatin improves these factors in a delayed manner associated with its cholesterol-lowering effects. Indeed, oxidized LDL induces P-selectin expression in human endothelial cells that are involved in mediating monocyte adhesion.⁵² This mechanism is involved in the downregulation of NO synthesis,⁵³ the inhibition of platelet plasma membrane Ca²⁺-ATPase,⁵⁴ and the modulation of intracellular signaling via Rho-kinase⁵⁵ and phospholipase A2⁵⁶ in platelets. Atorvastatin reduces the expression of platelet CD36 and LOX-1, which are oxidized LDL receptors, in association with decreased platelet-associated oxidized LDL, resulting in platelet deactivation.¹¹ Schafer et al.⁵⁷ also demonstrated that rosuvastatin reduces platelet activation by enhancing endovascular NO bioavailability in rats with chronic heart failure. Although the precise relationships among oxidized-LDL, platelet P-selectin and IL-6 remain unknown, some investigators have shown that they are involved in the pleiotropic effects of statins.

All patients in the present study with CAD were administered with aspirin, which is a potent antiplatelet agent that might have affected the platelet surface expression of P-selectin. Moreover, the levels of P-selectin at baseline and at 4 weeks in the control group were lower than those in the atorvastatin group, although the features of the participants including age, sex, lipid profile, and nature of disease did not significantly differ between the groups for reasons that remain unclear. Further studies of large populations including normal controls are required. We also did not investigate the short- or long-term effects of various doses or types of statins, including the incidence of CAD.

In conclusion, we demonstrated that atorvastatin lowers LDL-C and subsequently reduces MDA-LDL and platelet P-selectin that are associated with the reduction of IL-6 in patients with CAD and high levels of LDL-C. Atorvastatin might ameliorate the progression of atherosclerosis independently of its ability to decrease cholesterol.

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